Response to Reviewer Comments

# Reviewer 1

The article is devoted to the study of the role of the association of cholesterol and calcium in the blood in the development of cardiovascular diseases. The article made a strange impression on me. The authors analyzed data obtained on mice, including those on a high-fat diet. The authors compared data obtained in mice with data obtained in humans. Already in the Summary, as well as in the Discussion (for some reason, the authors called this section Conclusion), the authors themselves indicate that the results they obtained on mice fully correspond to the results obtained in many multicenter cohort studies on humans. It is known that cohort studies in humans are, today, the standard for obtaining results on risk factors and predictors of various diseases. At the same time, the results of animal studies help to understand the mechanisms of disease development. The authors do not talk about the mechanisms of development of cardiovascular diseases in their results. Then why did they do this research? To validate data from human cohort studies? It is very strange. I believe that the article and the purpose of the study need to be deeply rethought, to formulate the purpose in a different way, to recalculate and rewrite in a different non-scientific key.

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**Response Figure 1: Forrest plot of the associations between cholesterol and calcium in human epidemiological data.** A meta-analysis was performed on studies that evaluated the associations between calcium and cholesterol in a total of 88939 human subjects across 9 studies. In the case of several of these studies data was only presented stratified by subgroups so these are denoted with m, f, f-pm (post-menopausal), or m20-39/m40-69 (males sub-grouped by age). Random effects modelling shows a significant association between calcium and cholesterol with an estimated correlation of r=0.17 (95% CI 0.13-0.20).

We thank the reviewer for their thought-provoking comments about the premise of this research. While the reviewer is correct to point out that our results correspond with the human data, these human data are a) not appreciated in the literature and b) less rigorous in design than our mouse data.

To the first point, while many human epidemiological studies have suggested an association between cholesterol and calcium in blood, to our knowledge there is both not well appreciated in the field of lipidology. Our manuscript is the first to rigorously bring together these often-ignored findings and generalizes them not just to humans but also to experimental animals, reducing the potentiality of residual confounding. To our knowledge, while there have been no systematic reviews or meta-analyses of these human findings. To demonstrate the variability in the human data, and to clarify this point, we performed our own meta-analysis of these associations (Figure 1 of this response), summarizing studies that tested this association [1–9]. Note the overall heterogeneity of these results (I2=95%, p<0.01). Several other studies cited in our manuscript have also demonstrated significant positive associations, but were analyzed by quantile regression [10–13] or only reported LDL-C not cholesterol [14] so could not be integrated in this meta-analysis. We feel that the work reported in this manuscript in experimental animals is strongly supportive of this human data and we look forward to publishing this meta-analysis of the human calcium cholesterol associations separately.

In terms of experimental rigor, our approach has several advantages over the human observational studies including known and controlled diets, and environmental conditions. This is a major concern in human observational studies where populations with co-incident (and often unknown) behaviors and environments cannot be effectively disentangled. This less of a concern for our results. This is noted in the discussion on lines 188-191:

**We present data on a large number of mice roughly equally divided between sexes and two diets and find consistent results across all groups. We have exceptional control of confounders such as diets, environment, activity levels, and other exposures that could affect the interpretation of the human studies**

As an example, calcium and lipid intake vary widely among humans, and diets may be high in both calcium and lipids (or other factors) which could result in confounded human data. Furthermore, other environmental factors may correlate with diets high in calcium or lipids. This is noted as a limitation in most of the human studies cited above. This is much less of a concern in our studies where the diets are entirely controlled and are binary in nature. This reduces variability and increases the rigor of the association between calcium and cholesterol. To demonstrate the increased rigor of this design, the estimated association in humans is r=0.17 (Response Figure 1), but in mice it is 0.39 to 0.48 depending on the subgroup. While it is possible that the true association is stronger in mice, we believe that this is due to decreased dietary and environmental variability in mouse studies resulting in enhanced experimental power. The data in this manuscript increases the confidence in the overall hypothesis that calcium and cholesterol levels are related beyond those human studies.

Finally, the extension of these associations to experimental mouse systems (of which this study is the first demonstration) enables dissection of the causal mechanisms that both our team and the reviewer are eager to further understand. Without the knowledge that cholesterol and calcium levels are associated in mice, it would be premature to test how calcium regulates cholesterol (and vice versa) in mice. As such these data are critical to those future studies.

We decided not to extend our findings to direct implications on cardiovascular disease, as this was not observed in our animal models. This is added as a limitation on line 211-213:

**Finally, as cardiovascular disease is extremely rare in mice of this age we did not assess cardiovascular disease as an endpoint in this study.**

# Reviewer 2

Line 63: it is not clear which details are included in the previous publication. Please specifiy:

Reference 9 refers to the original paper describing these mice. We have described all the experimental procedures and details that we feel are pertinent in this manuscript’s methods section but wanted to provide this reference for other not anticipated details the reader may want.

Some typos or grammar errors are present all along the text**:**

The manuscript has now been gone through and typos and grammatical errors have been identified and corrected.

Data have to be reported as mean and standard deviation. Standard error is not the correct way to express the variability within the groups:

We revised our data presentation to show standard deviation as requested. This affected Figure 2C, Supplementary Figure 1 and multiple locations in the results section.

Figure 1: the sum of reported n in the box is 818 and not 822 as stated in the legend. I could not find any information on the body weight:

Chart, scatter chart

Description automatically generated

**Response Figure 2: Associations between body weight and cholesterol.** Scatter plot of mouse body weight and cholesterol levels stratified by sex and diet.

As for body weight, this is an excellent point. First body weight was a predictor of total cholesterol, and was the first pruned branch of the regression tree presented in Figure 1C, meaning that it was the next best predictor after diet, triglycerides and calcium levels, however based on our cross-validation it was removed. To answer this specific question of the role of body weight on cholesterol, we analyzed the associations between cholesterol and body weight and found an significant association between these variables, much of which is driven by the obesegenic high fat high sucrose diet (Response Figure 2). After controlling for sex and diet, we find that a 1g increase in body weight causes a modest but significant 0.85 +/- 0.16 mg/dL increase in cholesterol (p=6.2 x 10-8). As such there is an effect, but it is smaller than the effects of sex and calcium after diet is controlled for.

To address how much body weight (as opposed to diet) affected the relationship between calcium and cholesterol we performed stepwise modeling of calcium, sex, and body weight, comparing beta coefficients and p-values for the effects of calcium on cholesterol (Response Table 1). Our interpretation of these data is that body weight does partially mediate the relationship between cholesterol and calcium, as the beta coefficient for that relationship is mildly attenuated. This was also true when diet is included as a covariate as explained in the results section. In other words, the relationship between cholesterol and calcium remains substantial and significant even when accounting for differences in body weight.

**Response Table 1: Stepwise multivariate analysis of calcium and cholesterol and effects of body weight.** Stepwise models were constructed adding in sex and body weight as covariates with the beta coefficient for the effects of calcium and cholesterol and the p-value for that term indicated.

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| --- | --- | --- |
| **Model** | **calcium** | **p-value** |
| cholesterol~calcium | 14.37 | <1 x10-15 |
| cholesterol~calcium+sex | 14.55 | <1 x10-15 |
| cholesterol~calcium+sex+body.weight | 12.90 | <1 x10-15 |

The n of 822 was from a previous version of the dataset, where the n of 818 is from the updated dataset. This includes the 840 mice for which we had cholesterol and calcium data, but 22 mice that were omitted in the regression trees due to missing values in other variables. This is now clarified in the revised Figure 1 and 2 legends on lines 409 and 414.

To quantify this effect, we performed a bootstrapping-based causal mediation analysis described in [15]. We used the sex-moderated associations between cholesterol and calcium to estimate the proportion of the effect mediated by differences in body weight using 1000 simulations. As a result of this analysis we estimate that differences in body weight mediate approximately 11% of the cholesterol-calcium relationship. In turn, this approximates that 88% of the calcium-cholesterol relationship is independent of body weight. Overall, our conclusion is that these data supports our prior conclusion that the calcium-cholesterol relationship is largely independent of diet and differences in body weight and body composition, though these analyses do suggest that this is not completely separate from changes in body weight or composition.

# References

Numbering is for the response document only. These numbers are different in the main manuscript file.

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